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Nanoscopic vibrations by bacteria adhering to surfaces

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Chapter 6

General discussion

When brought into contact with a medium of natural origin material surfaces tend to be contaminated by bacteria,[1] as occurring in areas ranging from soil remediation, marine fouling to modern industrial production and medicine.[2-7] The study of bacterial adhesion processes has been intensified during the past decade and led to a better understanding of the underlying adhesion principles.[8-10] An important area of research in this field is related to the attractive forces involved in the interactions between bacteria and substratum surfaces.[11] Many theoretical models and technical methods have been developed, trying to infer or directly measure the strength and character of the bacterial bond. For instance, force-distance measurements were done by atomic force microcopy and bacterial adhesion forces were inferred from release characteristics observed in parallel-plate flow chambers.[12-14] Furthermore visco-elastic properties of bacterial bonds were analyzed by quartz crystal microbalance with dissipation (QCM-D) monitoring.[15]

Interactions involving bacteria and substratum surfaces are extremely complex and need to be assessed by multiple techniques. The analysis of Brownian motion induced bacterial vibrations provides a novel, convenient and direct way to characterize the visco-elastic bond between the bacterium and the substratum.[16] The challenge of this thesis was to offer a method to measure the amplitude and frequency of bacterial vibrations and to interpret the results of this study under different conditions with the results of various established analysis techniques, like QCM-D, particulate microelectrophoresis and the associated DLVO theory. Efforts to understand the bacterial vibration have revealed that bacterial vibration on different surfaces reflect the characteristics of combinations of non-specific and specific interactions.

Brownian Motion of Bacteria

Analysis of vibration of adhering bacteria was used in this thesis as the main technology to unravel the binding mechanism of bacteria to a substratum. The origin of this bacterial vibration is Brownian motion of sessile bacteria.

To understand how Brownian motion affects the vibration of sessile bacteria we first relate this to Brownian motion of planktonic bacteria. Brownian motion of non-adhering planktonic bacteria is governed by their intrinsic velocity as a result of their thermal kinetic energy and the exchange of their energy with the thermally agitated molecules of the medium.[17] As a result bacteria will move in random directions and change directions on an extremely short time scale inversely related to the viscosity of the medium which determines the viscous drag.[18] Because the movements are random and take place in all directions, the net displacement is zero. However, the mean quadratic displacement is non-zero and increases linearly with time, which is the basis of particle diffusion.

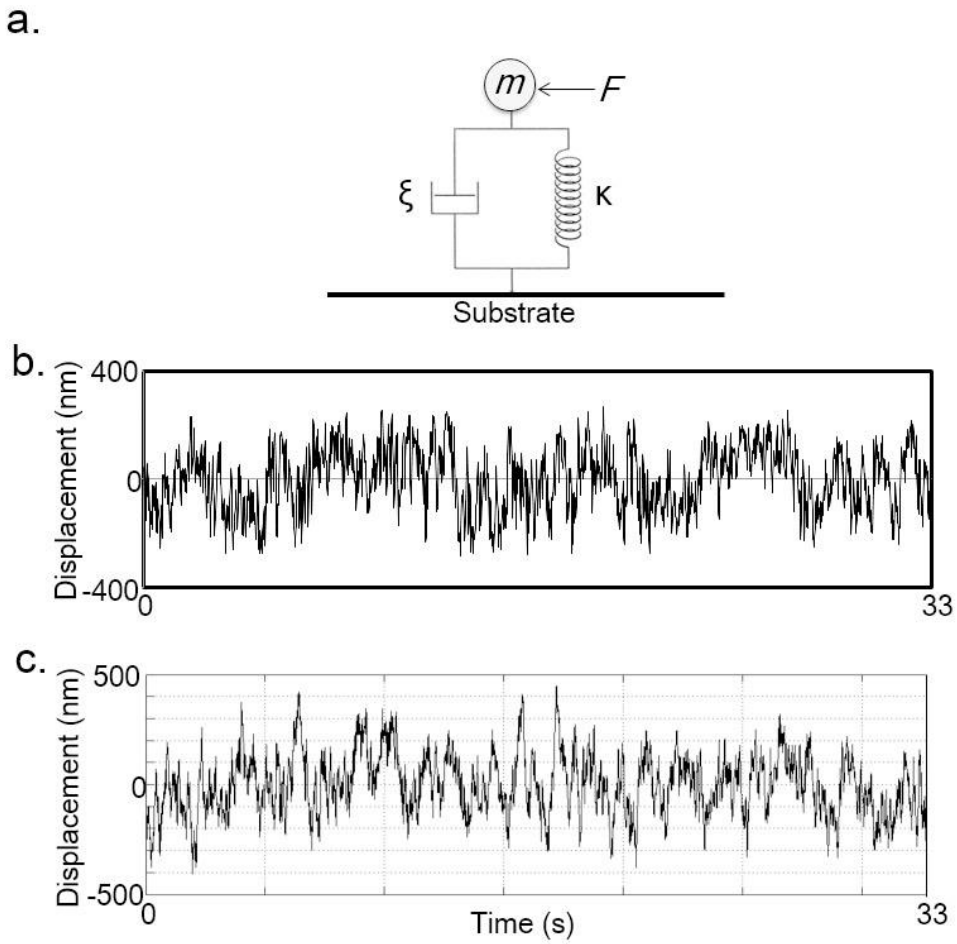

Figure 1. (a) Schematic representation of a bacterium connecting to the substratum surface with dashpot and spring.

(b) Example of time series of vibration displacement as a function of time for *S. mutans* ATCC25175 in adhesion buffer (0.57 mM) under static condition.

(c) Simulated Brownian motion displacement of a mass connected to a spring and a dashpot by Matlab. For the spring constant we used 1.2×10^{-5} N/m, damping coefficient 2×10^{-6} N/(m/s), mass 5×10^{-16} Kg. The driving force was simulated by a random generator, which exposed the mass to a random force in between -200 pN and 200 pN with a frequency of 10kHz. The simulation only involved vibrations in one dimension.

In case bacteria are adhering to a surface, they are not free to move. When we consider an adhering bacterium as a damped forced oscillator system (comprising a mass, m, connected to a spring with spring constant, k, and a dashpot with damping factor, ξ, exposed to a random force, F, representing interactions with molecules with a frequency ν, see Figure 1a) the bacterium experiences an additional potential

elastic energy from the bacterial bond with the substratum, which retracts the bacterium when it tries to move too far from the surface. Random Brownian motion still remains, but the mean quadratic displacement stays constant when averaged over a long time. When we simulate random Brownian motion of a bound bacterium as represented by this model (as was done in a simple Matlab program) we were able to generate similar random motion patterns (with respect to frequency and amplitude) as was observed from bacteria, as can be seen in Figure 1b and c. This may serve as an indirect prove that the bacterial vibrations are originally Brownian movements of bound bacteria and that the model might be useful in the future to gain more quantitative values for the bond strength and viscoelasticity constants of the bond under various conditions.

Characterization of the Bacterial Bond to a Substratum

One of the main phenomena discussed in Chapter 2 was the effect of the ionic strength on the vibration amplitude of attached bacteria. We demonstrated that the ionic strength has a large influence on the vibration amplitude of bacteria. This was explained in terms of the persistence length of the polymer chains in the outermost bacterial cell surface. The persistence length increases with decreasing ionic strength of the suspending fluid, and leads to expansion and reduced damping (according to QCM data) of the polyelectrolyte network, ultimately resulting in high vibration amplitudes. Moreover the bond appeared to behave as a non-linear elastic matrix, because stretching of the bond by switching on fluid flow, reduced the vibration amplitude and as result reflects an increasing spring constant. The non-linear behaviour can be explained by a redistribution of the polymer chains involved in the bond as was earlier observed by Mc Connel *et al.*[19] They found that microfibers of the abdominal artery of a lobster aligned under stress. The more fibers are aligned with the direction of stress, the greater the load it is able to carry and the stiffer the matrix. Despite its non-linear elastic behavior the bond characteristic remained reversible, however: switching off the flow resulted in higher vibration amplitudes again reflecting reduced spring constants.

This remarkable reversibility raises the question whether the effect of ionic strength on the visco-elastic properties is reversible as well, because this would further corroborate the model of a poly-electrolyte network.

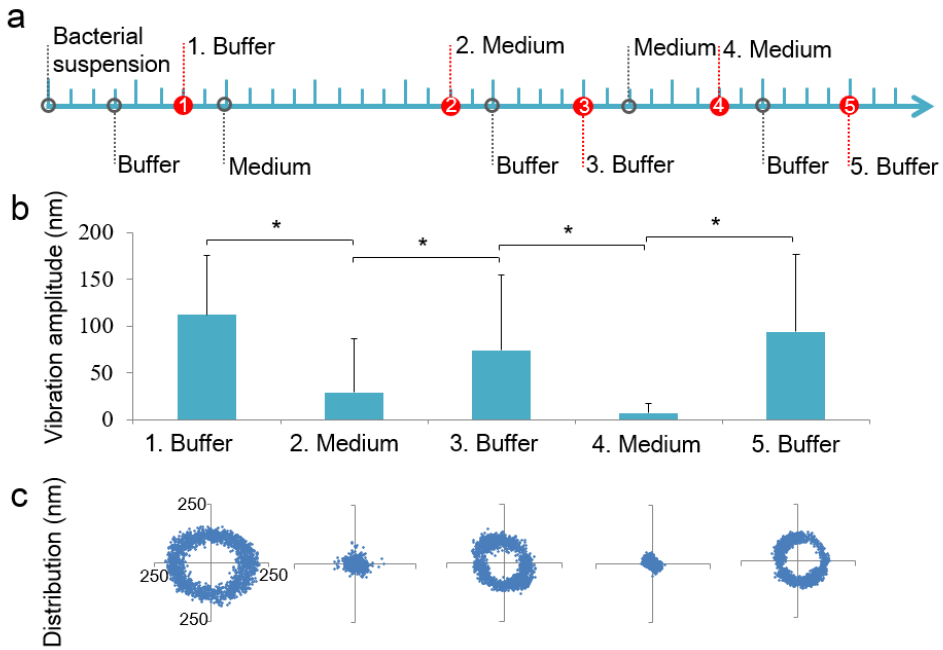

Figure 2. (a) Timeline of the procedure for *S. mutans* IB03987. Regularly spaced ticks indicate quarters of an hour. Red numbered bullets indicate the start of an observation. Numbers coincide with the number of the graphs beneath. The experimental testing began with a pre-adhesion from a bacterial suspension, followed by rinsing with adhesion buffer. Measurements were taken 15 min after arresting the flow. After that, full medium (BHI) was perfused through the system for 2 h to help with cell activity. Subsequently, medium without bacteria was perfused for 15 min in order to remove all planktonic bacteria. Again, measurements were taken 15 min after arresting the flow. Subsequently, this procedure was repeated for another three times with buffer and medium.

(b) Vibration amplitudes of bacteria adhering to glass surfaces after buffer and medium treatment, at points in time corresponding with the numbers in the upper panel.

(c) Position maps of the same adhering bacterium at various time points, which corresponding to panel b.

Therefore experiments were carried out with an alternating buffer and medium as is summarized in Figure 2a. Although it is difficult to measure the ionic strength of the medium exactly, the electric conductivity of medium (18.5 ms) is much higher than the adhesion buffer (0.135 ms), which indirectly proves that the ionic strength of the medium is higher than the adhesion buffer. Results of the vibration experiments show high vibration amplitudes in adhesion buffer (0.57 mM) and a significant decrease in medium due to the high ionic strength of the medium (Figure 2b) as expected. Subsequently, adhesion buffer was perfused again for 30 min, resulting in higher amplitudes again (Figure 2b and c), consistent with previous results using the same buffer. Accordingly, the change in persistence length of the polymer chains due to changes of the ionic strength is fully reversible.

In Chapter 3 a slightly different approach was followed to characterize the bacterial bond with the substratum. In the first place the system was far more complex by the introduction of proteins adsorbed onto the surface, showing that firm attachment to soft protein layers may contribute to larger bacterial vibration amplitudes as a result of the large motional freedom of the protein chains they attach to. But in the absence of proteins, as was done earlier in Chapter 2, a similar effect of ionic strength on vibration amplitudes was interpreted in terms of the DLVO theory instead of an explanation in terms of persistence length changes and softening of protein chains in the outermost bacterial cell layer. It is suggested that the depth of the secondary interaction minimum is playing an important role (See Chapter 3, Figure 1f) because vibration amplitudes increased with decreasing depth of the secondary interaction minimum, assessed on basis of zeta potential and streaming potential data.

Although in Chapter 3 bacteria involved (*S. mutans, S. aureus*) were different from those used in Chapter 2 (*S. salivarius, S. epidermidis* and *S. aureus*) the binding mechanisms on bare glass will not differ very much. It is, however, hard to say which of the interpretations is the most realistic one (the one based on persistence lengths or on DLVO) and perhaps explanations could have been interchanged in both chapters. Most probably both interpretations are abstractions of or conventions to describe the same phenomena or simply act on top of each other. Both are plausible by realizing that zeta potentials (on which the mechanism in Chapter 3 is based) are largely influenced by the softness and water permeability of the outermost bacterial cell surface, which is highly dependent on the ionic strength as was the main argument in Chapter 2 to explain the dependence of bacterial vibrations on ionic strength. Both interpretations, however, do show that bacterial vibrations are a highly sensitive parameter for characterizing the bond between bacterium and the substratum. Apparently, a quantitative theory relating amplitude and frequency in terms of thermodynamic or physico-chemical theories will be highly complex but may provide us with a lot of novel insights in the character of the bacterial bond.

Live and Dead Cells: Do They both Vibrate?

In Chapter 4 vibration analysis was explored again to study the relationship between the bond strength and vibration amplitudes. We demonstrated that amplitudes of bacterial vibration originating from Brownian motion, correlate with the rate of bacterial detachment on bare glass surfaces. Apparently the maximum displacement a bacterium is allowed by the binding forces is directly related to the chance that bacteria eventually escape from the surface under the force of an air-liquid interface passage. This could even be shown on substrate with conditioning protein (saliva and fibronectin) films (see Figure 3) where bacteria obtain extra motional freedom from protein chains where they attach to.

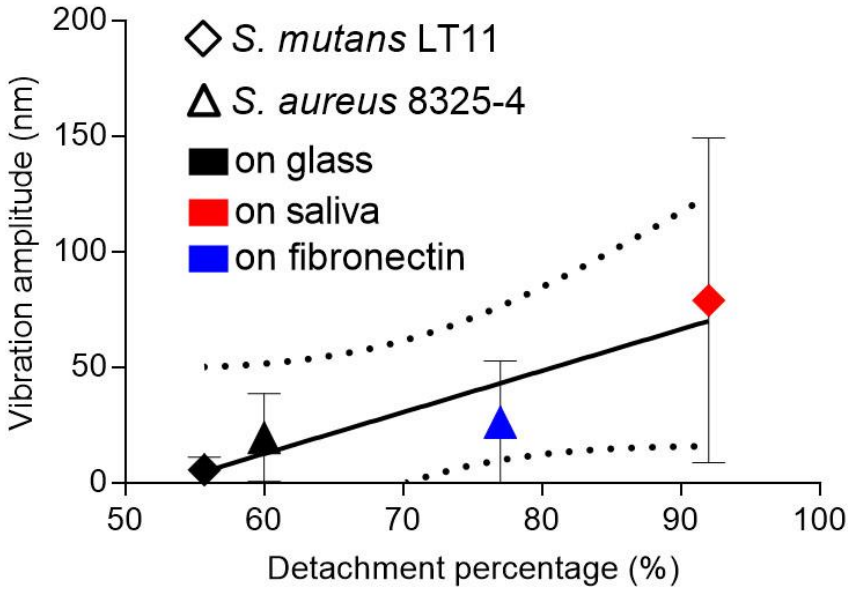

Figure 3. Vibration amplitudes of adhering bacteria as a function of detachment percentages stimulated by a passing liquid-air interface observed for two bacterial strains on saliva and fibronectin coated and uncoated glass (linear correlation coefficient 0.87). For vibration amplitude measurements, three experiments were done with separately cultured bacteria, each experiment comprising a minimum of 10 randomly selected adhering bacteria. The solid line shows the prediction line of the correlation based on an assumed linear regression and the dashed lines represent the 95% confidence interval. Vertical error bars indicate standard deviations over 30 different bacteria from three experiments with separately grown cultures.

In addition, our results showed that cationic detergents dramatically increased the bacterial adhesion force due to stiffening of the adhesive bond, reflected in low vibration amplitudes. For example, after chlorhexidine (CHX) treatment, significant upward wave number shifts in IR absorption band were found which suggests an increase in the stiffness of polysaccharides (Chapter 4). This may be caused by both adsorption and absorption of positively charged moieties from the CHX in the bacterial cell wall. These changes result in stronger binding of the bacteria to the surface.

As with chlorhexidine, most cationic detergents used as a mouthrinse are bactericidal, which means that the strong adhesion forces resulting in small vibration amplitudes are accompanied by cell death. Although it was stated in Chapter 4 that being dead or alive has no impact on Brownian motion-induced vibrations it is worthwhile to reconsider this, since vibrations of live cells may also originate from autonomous movements of bacteria, since bacterial membranes contain ionic pumps that may give rise to small motions closely related to the bacterial metabolism.[20] Longo *et al.* were able to sense these vibrations by using an atomic force microscope cantilever which picked up these vibrations and showed that exposure to the antibiotic

ampicillin resulted in annihilation of the vibrations, that did not return after flushing with buffer.[21] The mechanism of action of ampicillin is inhibition of cell wall synthesis, causing a metabolic shock from which susceptible bacteria do not recover.[21] No cell wall leakage is expected upon exposure to ampicillin in contrast to CHX. In order to find out whether these cell wall vibrations may act as an indicator of bacterial cell death and could be sensed in our vibration analysis as well, we compared vibration of *S .mutans* IB03987 on glass before and after exposure to 50 μg/mL ampicillin in the medium and 0.2 % m/v chlorhexidine digluconate (CHX) (see Figure 4). Both concentrations were higher than the MBC found for this strain. It therefore may be assumed that the bacteria were killed by both the antibiotic and the CHX.

For the antibiotic treated bacteria, there was no significant difference between the vibration amplitudes in medium with and without antibiotic. After flushing with adhesion buffer with low ionic concentrations, a significant increase in the vibration amplitudes was found towards similar values as before the exposure to antibiotics.

However, for the CHX treated bacteria after adhesion buffer rinses, the vibration amplitudes remained low (see Figure 4c). It suggests that the antibiotic was not able to permanently reduce the amplitude of the vibrations, whereas CHX permanently changed the bond constituents and its distribution. Apparently cell death itself is not affecting bacterial vibrations.

Although the present analysis was limited to only one antibiotic and one strain the results might be generalized to the conclusion that indeed cell death cannot be inferred from vibration analysis alone. But if an episode of antiseptic or antimicrobial exposure is followed by a passage of buffer and vibrations are not recovered, the permanent lack of Brownian motion may indicate that the bacterium is severely disrupted and cell constituents have permanently been changed to affect the bacterial bond.

CONCLUSIONS

This thesis shows that the analysis of vibrations of adhering bacteria provides novel insights in the characteristics of the bacterial bond. By comparing the vibration amplitudes at different ionic strengths (Chapter 2 and this chapter), surface structures (Chapter 2), protein films (Chapter 3), antimicrobials (Chapter 4 and this chapter) and cell death (this chapter), basic understanding was obtained how to interpret bacterial vibrations in a way that helps to unravel the complex nature of bacterial adhesion. Most of the chapters only exploit changes in vibration amplitudes,

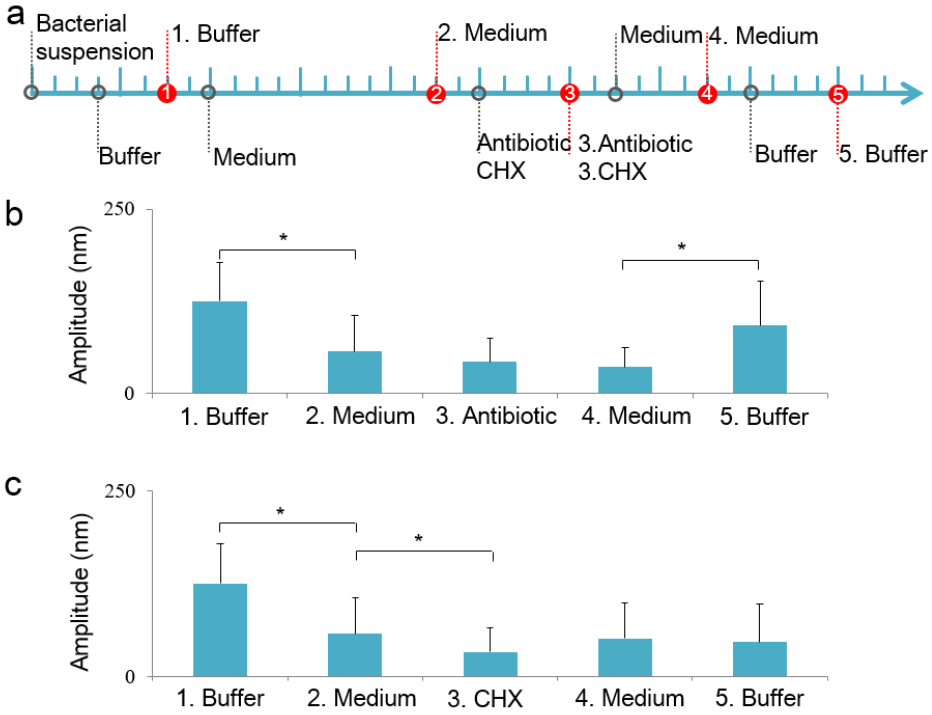

Figure 4. (a) Timeline of the procedure for *S. mutans* IB03987. Regularly spaced ticks indicate quarters of an hour. Red bullets indicate the start of an observation. Numbers coincide with the number of the graphs beneath. The flow protocol was similar as described before in Figure 2 but was extended by the passage of buffer after the exposure to medium and antimicrobials. After measurement in medium, the antibiotic ampicillin in medium or CHX was perfused for 30 min and static conditions were created for 15 min before and during the measurement.

(b) Vibration amplitudes of *S. mutans* IB03987 adhering on a bare glass substratum before and after treatment with antibiotic in various conditions.

(c) Vibration amplitudes of *S. mutans* IB03987 adhering on a bare glass substratum before and after treatment with CHX in various conditions.

whereas frequency and correlation time has been left out so far (except for a short discussion in Chapter 2). New investigations applying this technology may further exploit these vibrational characteristics, possibly also by increasing the frame rate of the camera. Frequency and correlation analysis may particularly of interest in extreme situations like the first arrival and attachment of a bacterium from flow or the spectroscopic changes during a gradual increase of shear forces. Therewith a novel analysis tool, perhaps best designated as “vibrational spectroscopy”, has been introduced that complements instruments that are already established in this field, like Atomic Force Microscopy and QCM-D.

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